Optimizing Pemetrexed-Gemcitabine Combination in Patients with Advanced Non-small Cell Lung Cancer

A Pharmacogenetic Approach

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Introduction: The pemetrexed-gemcitabine combination is effective in patients with non-small cell lung cancer (NSCLC). Preclinical data suggest that pemetrexed may synergistically interact with gemcitabine by enhancing the expression of human equilibrative nucleoside transporter 1 (hENT1) and deoxycytidine kinase (dCK), increasing the uptake and intracellular activation of gemcitabine. A pharmacogenetic approach was adopted to evaluate hENT1 and dCK expressions in humans and to identify the potential best time interval to administer gemcitabine after pemetrexed in patients with advanced NSCLC.

Methods: The dCK and hENT1 expressions, examined by quantitative real-time polymerase chain reaction, were analyzed during each cycle before and at 1, 2, 4, 6, 24, and 48 hours after pemetrexed administration. The relative differences from baseline to each planned time, for peak values and for the relative difference at peak, were measured.

Results: Nineteen patients were treated with pemetrexed single agent (500 mg/m^2 every 15 or 21 days). Quantitative real-time polymerase chain reaction analysis revealed a statistically significant ($p < 0.001$) biphasic increase in both hENT1 and dCK genes at 1 to 2 and 24 to 48 hours after pemetrexed administration.

Conclusions: This is the first evidence of dCK and hENT1 induction by pemetrexed in humans, suggesting that the pemetrexed→gemcitabine combination should be optimized by the administration of gemcitabine 1 to 2 or 24 to 48 hours after pemetrexed. These results support further studies to validate the role of dCK/hENT1 in vivo modulation for the optimization of gemcitabine-pemetrexed combination in patients with NSCLC.

Key Words: dCK, hENT1, Gene expression, Pemetrexed, Gemcitabine.

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Chemotherapy with platinum-based doublets represents the backbone of treatment for advanced non-small cell lung cancer (NSCLC),[1] but the lack of balance between therapeutic activity and toxicity supports the research of novel platinum-free combinations, together with the availability of third-generation drugs. Among these, pemetrexed has shown to be active in NSCLC, with a suggested major antitumor activity in nonsquamous tumor types,[2] and having a favorable toxicity profile,[3] which makes this drug an attractive agent for combined regimens with other active cytotoxics.

Pemetrexed is a novel agent that acts on several folate metabolism enzymes, which are involved in DNA synthesis and cell death control.[4–6] In particular, deoxycytidine triphosphate depletion and glycinamide ribonucleotide formyltransferase inhibition induced by pemetrexed might enhance the expression of the key genes involved in gemcitabine transport and metabolism, as reported in several preclinical cancer models, including NSCLC cells.[7,8]

Gemcitabine is a deoxycytidine analog, which activity depends on the ribonucleotide reductase enzyme inhibition and on drug incorporation into the DNA during the S phase. Because of its hydrophilicity, gemcitabine does not cross the

cellular membrane by diffusion, and it is transported into cells by the human equilibrative nucleoside transporter 1 (hENT1). After cellular uptake, gemcitabine must be phosphorylated to its active metabolites by the enzyme deoxycytidine kinase (dCK). These two passages are rate-limiting steps for gemcitabine activation, and the higher expression of dCK and hENT1 has been associated with gemcitabine sensitivity in NSCLC cells. Several in vitro experiments showed that the sequence pemetrexed→gemcitabine is more active than gemcitabine→pemetrexed.[7,9,10]

The synergistic interaction of pemetrexed-gemcitabine was associated with the induction of the dCK and hENT1 expressions by pemetrexed. These preclinical data are consistent with the results of a phase I trial[11] and a randomized, phase II trial testing three different schedules of pemetrexed and gemcitabine in combination.[12] This evidence was also supported by a recent phase II study in patients with NSCLC, where the gemcitabine→pemetrexed combination was minimally active, with a high incidence of grade 3 or 4 toxic effects, requiring frequent dose adjustments.[13] Nevertheless, definitive evidence of the synergistic modulation of gene expression in humans is missing, and no information is currently available regarding the optimum time interval between these two drugs. Answering these questions by the "clinical evidence-based methodology" should require a multiarm, randomized, controlled trial, which, if designed in the absence of proper information, would present with inherent disadvantages such as the high cost involved and the need for a large sample size. To explore whether a synergistic interaction between pemetrexed and gemcitabine is reproducible in humans and to determine the most effective interval between the two drugs, a pharmacogenetic approach has been adopted in this study. Therefore, both the dCK and hENT1 expressions have been examined as a surrogate end point, using freshly isolated lymphocytes, at various time intervals, up to 48 hours after pemetrexed administration, in patients with advanced NSCLC.

PATIENTS AND METHODS

Patients with histological or cytological evidence of stage IIIB and stage IV NSCLC and not suitable for platinum-containing regimens or pretreated with regimens not containing gemcitabine or pemetrexed were eligible for this study. Other eligibility criteria included age ≥18 years; Eastern Cooperative Oncology Group performance status (PS) ≤2; estimated life expectancy of at least 12 weeks; adequate bone marrow (platelets $\geq 100 \times 10^9$ cells/L, absolute neutrophil count $\geq 1.5 \times 10^9$ cells/L, and hemoglobin ≥9 g/dl); hepatic (total bilirubin, ≤1.5 times the upper limit of normal, alkaline phosphatase, aspartate transaminase, and alanine transaminase ≤3.0 × upper limit of normal. Aspartate transaminase and alanine transaminase ≤5.0 × upper limit of normal were acceptable if documented liver metastases were present.); and renal (calculated creatinine clearance, ≥45 ml/min, using the standard Cockcroft calculated creatinine clearance formula) functions.

Exclusion criteria included symptomatic or active brain metastasis, diagnosis of significant cardiac disease, pregnant or nursing women, radiation therapy to more than 25% of bone marrow, inability to discontinue therapy with aspirin at doses more than 1.3 g/d or nonsteroidal antiinflammatory drug therapy for a 5-day period, clinically significant pleural or peritoneal effusions, and diagnosis of myocardial infarction or significant cardiac disease. Patients were excluded from the study also if they had received treatment within the last 30 days with a drug (not including study drug) that had not received regulatory approval for any indication at the time of study entry. This pilot study complies with the ethical standards laid down in the 1964 Declaration of Helsinki. Ethics committee approval was taken, and written informed consent was obtained from all patients enrolled in the study (H3E-IT-S105).

Pemetrexed was supplied as lyophilized powder by Eli Lilly & Company and reconstructed in sodium chloride solution before use. Patients received pemetrexed at a dose of 500 mg/m^2 as a 10-minute intravenous infusion at one of the following schedules: once every 15 days (before amendment, $n = 12$) or once every 21 days (after amendment, $n = 7$).

Dexamethasone (4 mg), or equivalent corticosteroid, was given orally twice a day, starting the day before and continuing until the day after study drug administration. Folic acid (450 μg) was administered orally daily beginning 1 to 2 weeks before first pemetrexed administration and for the entire study duration. Vitamin B12 (1000 μg) was given as intramuscular injection every 9 weeks starting 4 weeks before the first dose of pemetrexed and continuing until 3 weeks after discontinuation of study therapy. Treatment was continued for a maximum of six of eight cycles (before/after amendment) or until documented disease progression, unacceptable toxicity, or patient refusal.

Isolation of Peripheral Blood Mononuclear Cells

During the first three cycles, 15 ml of blood was withdrawn from a forearm peripheral vein at 1-, 2-, 4-, 6-, 24-, and 48-hour intervals after pemetrexed administration. Whole blood was collected using BD-Vacutainer CPT, and the separation of mononuclear cells was performed on a Lymphoprep Separation Medium (Gibco Biology Research Laboratories) following manufacturer's instructions. Total cellular RNA was then isolated from mononuclear cells using Qiagen RNeasy Mini kit.

Quantitative Real-Time Polymerase Chain Reaction

RNA (50–500 ng) was reverse transcribed with a Script complementary DNA (cDNA) Synthesis kit (BioRad). cDNA amplification was performed on a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Forward and reverse primers and probes were designed with Primer Express 2.0 based on dCK gene sequence obtained from GenBank, whereas primers and probes for hENT1 were obtained from Applied Biosystems Assay-on-Demand products. Validation experiments were carried out with cDNA obtained from quantitative polymerase chain reaction human reference total RNA to show that the efficiencies of amplification of the target and the reference genes glyceraldehyde 3-phosphate dehydrogenase were approximately equal. Reac-

tions were performed in triplicates, and polymerase chain reaction (PCR) reagents and primers, without RNA as reaction-negative controls, were added to each real-time PCR assay. The coefficient of variation was less than 2% for all replicates.

To support the study rationale, quantitative real-time PCR analysis was also performed in healthy subjects ($n = 6$, four men and two women; mean age: 34 years, 30–44 years) in absence of diabetes, cardiovascular, inflammatory or autoimmune disease, cancers, obesity, or any other chronic disease in their clinical history. This evaluation was spontaneously performed by investigator site, earlier than study initiation, to assess baseline values (and potential picks) of dCK and hENT1, at sequential time intervals, without pemetrexed and related premedication exposure. An informed consent was provided by all the volunteers.

Statistical Methods

Summary statistics were calculated for original values of dCK and hENT1, for the relative differences from baseline to each planned time, for the peak values, and for the relative difference at peak, by cycle. The 95% confidence interval for the mean peak value and for the relative difference at mean peak was calculated. A multivariate repeated-measures analysis of variance on dCK and hENT1 values was performed to test the main effect of cycle, the main effect of time, and the interaction effect of cycle-x-time. Descriptive statistics were provided for objective tumor response.

RESULTS

Patient Characteristics

From September 2006 to March 2008, 19 patients with histologically or cytologically proven IIIB and IV NSCLC were enrolled (Table 1).

All patients completed at least one cycle of treatment and were included in the intention-to-treat (ITT) population. After the enrolment of the first 12 patients, the pemetrexed schedule was changed, from a 2-week to a 3-week regimen, adopted in further seven patients.

Because of the higher rate of toxicities requiring recurrent delays in pemetrexed biweekly administration, the protocol was amended, to change the biweekly schedule and restore the standard one (q21 schedule). What needs to be highlighted is that as this is a pilot study, with the aim of assessing both dCK and hENT1 expressions after pemetrexed administration only, patient selection was not so strict, and also patients with comorbidities and poor PS were enrolled.

Out of 19 enrolled patients, two (10.5%), two (10.5%), one (5.3%), four (21.1%), two (10.5%), two (10.5%), three (15.8%), three (15.8%) patients received one, two, three, four, five, six, seven, and eight cycles, respectively.

One patient with baseline total bilirubin above the required limit was enrolled with sponsor approval and was excluded from the per-protocol population ($n = 18$).

Six (31.6% of total) subjects completed the study, whereas 13 (68.4%) were prematurely discontinued. Adverse events (AEs) (seven patients, 36.8%) and disease progression (four patients, 21.1%) were the causes of early withdrawal.

TABLE 1. Demographic and Disease Characteristics

Continuous Variables	Mean ± SD
Age (yr)	69.2 ± 8.9
Weight (kg)	68.7 ± 13.1
Height (cm)	166.4 ± 10.1
Categorical Variables	***N* (%)**
Gender	
Male	11 (57.9)
Female	8 (42.1)
History of previous antitumor treatment	
Patients pretreated	13 (68.4)
Patients underwent surgery	14 (73.7)
Stage of disease	
IIIB	4 (21.1)
IV	15 (78.9)
Histological type	
Adenocarcinoma	11 (57.9)
Squamous cell carcinoma	6 (31.6)
Other	2 (10.5)
Histological grading	
G1: well differentiated	2 (10.5)
G2: moderately differentiated	3 (15.8)
G3: poorly differentiated	5 (26.3)
Not performed	9 (47.4)
ECOG performance status	
0	5 (26.3)
1	11 (57.9)
2	3 (15.8)

SD, standard deviation; ECOG, Eastern Clinical Oncology Group; G, grading.

Two patients (10.5%) discontinued the study due to Investigator's decision for lack of drug efficacy.

Expression of dCK and hENT1 in Patients After Pemetrexed Administration

In the entire study, the results of dCK and hENT1 peak values (mean ± SD) were 0.99 ± 0.03 and 0.93 ± 0.03, respectively, whereas the relative differences at dCK and hENT1 peak were 7.82 ± 2.15 and 8.54 ± 3.12, respectively. Mean dCK percent differences at peak (±SD) were 6.65 ± 2.33 at cycle1, 6.68 ± 2.25 at cycle 2, and 6.93 ± 1.26 at cycle 3, whereas for hENT1, the percent difference at peaks was higher at cycle 2 (7.31 ± 3.12) than at the other two cycles, cycle 1 (5.63 ± 2.45) and cycle 3 (6.56 ± 1.66). The results of dCK and hENT1 expressions reported as mean values evaluated at the various interval times at cycle 1, cycle 2, and cycle 3 in the overall ITT population are listed in Table 2.

The repeated measures analysis of variance showed a statistically significant time effect ($p < 0.001$) in both genes expression. A statistically significant ($p < 0.0001$) increase from baseline was observed in mean values of the hENT1 and dCK expressions after 1, 2, 24, and 48 hours (irrespective of the cycle). The dCK mean values were smaller but significantly different also at 4 hours ($p = 0.030$), but the hENT1 mean values were not ($p = 0.333$). At 6 hours, expression levels were lower than baseline for both hENT1 and dCK

TABLE 2. Mean ± SD dCK and hENT1 Expressions Evaluated at Cycle 1, Cycle 2, and Cycle 3 in the Overall ITT Population

	dCK			hENT1		
	Cycle 1	Cycle 2	Cycle 3	Cycle 1	Cycle 2	Cycle 3
Baseline	0.92 ± 0.03	0.92 ± 0.03	0.92 ± 0.02	0.86 ± 0.03	0.86 ± 0.03	0.87 ± 0.02
1 h	0.96 ± 0.04	0.95 ± 0.03	0.96 ± 0.03	0.88 ± 0.04	0.89 ± 0.04	0.90 ± 0.03
2 h	0.96 ± 0.04	0.96 ± 0.04	0.97 ± 0.02	0.88 ± 0.04	0.90 ± 0.04	0.90 ± 0.04
4 h	0.93 ± 0.04	0.94 ± 0.04	0.92 ± 0.03	0.86 ± 0.04	0.88 ± 0.05	0.86 ± 0.03
6 h	0.92 ± 0.03	0.91 ± 0.04	0.91 ± 0.03	0.86 ± 0.04	0.86 ± 0.04	0.86 ± 0.02
24 h	0.96 ± 0.04	0.97 ± 0.03	0.96 ± 0.03	0.89 ± 0.04	0.91 ± 0.04	0.91 ± 0.02
48 h	0.97 ± 0.04	0.96 ± 0.04	0.97 ± 0.03	0.90 ± 0.04	0.91 ± 0.04	0.91 ± 0.02

ITT, intention to treat; SD, standard deviation; hENT1, human equilibrative nucleoside transporter 1; dCK, deoxycytidine kinase.

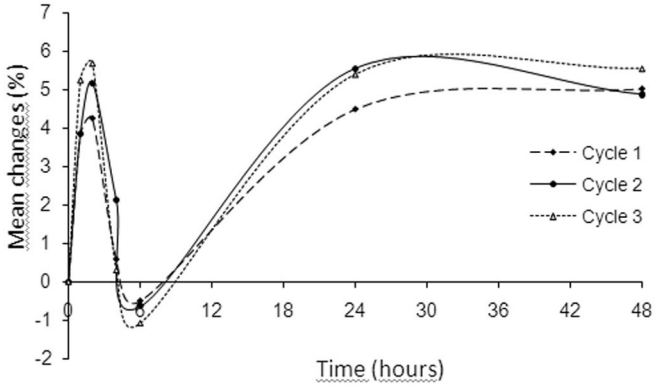

FIGURE 1. Mean changes (%) from baseline of deoxycytidine kinase (dCK) expression evaluated at cycle 1, cycle 2, and cycle 3 in the overall intention-to-treat (ITT) population.

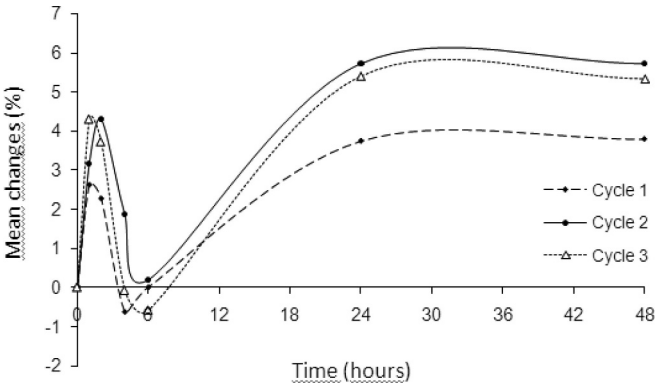

FIGURE 2. Mean changes (%) from baseline of human equilibrative nucleoside transporter-1 (hENT1) expression evaluated at cycle 1, cycle 2, and cycle 3 in the overall intention-to-treat (ITT) population.

values, but these differences were not statistically significant ($p = 0.849$ and $p = 0.166$, respectively). The cycle effect resulted statistically significant only in hENT1 expression ($p = 0.037$). The comparison of cycle 2 and cycle 3 versus cycle 1 showed statistically significant differences ($p = 0.014$ between cycle 2 and cycle 1, and $p = 0.016$ between cycle 3 and cycle 1) in hENT1 expression and not in dCK expression.

The mean changes, expressed as percentage, from baseline of dCK and hENT1 expressions revealed a significantly marked increase after 1, 2, 24, and 48 hours at any cycle, a smaller increase after 4 hours, and a nonsignificant decrease after 6 hours (Figures 1 and 2).

No substantial differences between the two schedules of administration were observed in the dCK and hENT1 expressions measured in any cycle. Results from the per-protocol population were consistent with those observed in the ITT population.

The quantitative real-time PCR analysis performed in the healthy subjects revealed that both the dCK and hENT1 gene expression were unchanged over the predefined time intervals (throughout 48 hours). Baseline dCK (0.93 ± 0.02) and hENT1 (0.83 ± 0.02) values in the patient population were similar to baseline levels in healthy subjects.

Evaluation of Tumor Response (RECIST Criteria)

At the end of the study, one patient (5.3%) had partial response, six (31.6%) had confirmed stable disease, eight (42.1%) did not have confirmed stable disease (not confirmed within 6 weeks after the first observation), three (15.8%) had progression of disease, and one patient died prematurely. Overall, seven were responders and 12 were not. The response rate was 57.1% in patients treated with a 3-week regimen and 25.0% in those treated with the 2-week regimen.

Safety

All AEs assessment was performed during study treatment.

AEs were reported on a per-subject basis. This means when a patient experiences the same AE repeatedly, the event is counted once only.

Five patients (26.3%) experienced serious AEs (SAEs). Out of five patients (26.3%) who experienced SAEs, only one patient reported drug-related SAEs (bleeding and thrombocytopenia). All other SAEs should be considered as a consequence of tumor progression.

Drug-related AEs were reported in 18 patients (94.7%), whereas in 16 patients (84.2%), the dose was modified or the treatment prematurely interrupted due to AEs. Grade 3 and grade 4 AEs are listed in Table 3.

TABLE 3. Grade 3/4 AEs

	q15 Schedule (n = 12)		q21 Schedule (n = 7)	
	G 3	G 4	G 3	G 4
Hematologic, *n* (%)	5 (41.7)	1 (8.3)	0 (0)	1 (14.3)
Neutropenia	2 (16.7)	0 (0)	0 (0)	1 (14.3)
Thrombocytopenia	1 (8.3)	0 (0)	0 (0)	0 (0)
Leukopenia	2 (16.7)	1 (8.3)	0 (0)	0 (0)
Nonhematologic, *n* (%)				
Constipation	1 (8.3)	0 (0)	0 (0)	0 (0)
Diarrhea	1 (8.3)	0 (0)	0 (0)	0 (0)
Asthenia	1 (8.3)	0 (0)	1 (14.3)	0 (0)
Transaminase alteration	1 (8.3)	0 (0)	2 (28.6)	0 (0)

G, grade; AE, adverse event.

As the primary objective of this pilot study is to assess and evaluate both dCK and hENT1 expressions after pemetrexed administration, patient selection was not so tight, and poor PS patients or with comorbidities were also enrolled. That is probably the reason why a higher toxicity rate was observed.

Therefore, as recent evidences documented that patients with locally advanced or metastatic NSCLC could benefit from pemetrexed given on a 3-week schedule, with a low incidence of hematological and nonhematological toxicities,[14,15] a protocol amendment was issued to change the initial dose regimen (q15) to a 3-week schedule (q21), which applied to the last seven patients.

DISCUSSION

The pemetrexed-gemcitabine combination seemed to be effective in NSCLC.

The schedule pemetrexed followed by gemcitabine seems to be more active than the sequence gemcitabine→pemetrexed. This evidence is supported by in vitro experiments[7,9,10] and by a randomized, phase II study, in patients with NSCLC.[12] The schedule with pemetrexed followed by gemcitabine on day 1 and gemcitabine on day 8, with a confirmed response rate of 31%, met the protocol-defined efficacy criteria, whereas the schedule with gemcitabine on day 1 and pemetrexed followed by gemcitabine on day 8, with a confirmed response rate of 16%, did not.[12] Similarly, a recent trial[13] evaluating the activity of gemcitabine followed by pemetrexed in patients with advanced NSCLC found an overall response rate of 13%, with a similarly disappointing progression-free survival.

Nevertheless, a recent phase II trial to assess the objective response rate and safety of first-line biweekly gemcitabine and pemetrexed in patients with NSCLC older than 70 years was closed after only nine patients due to unacceptable toxicity.[16] Although other studies showed that the pemetrexed-gemcitabine schedule is well tolerated in elderly patients,[17–20] particularly frail patients can experience an excess of toxicities with a 2-week regimen, as we observed in our trial, where the protocol was amended to change the biweekly schedule and restore the standard one (3-week regimen).

Therefore, pemetrexed and gemcitabine administration should be further investigated. Moreover, definitive evidences of the possible mechanisms underlying their synergistic antitumoral activity in humans are still missing, and no information is available regarding the optimum time interval between the administrations of these two drugs.

In this study, a pharmacogenetic approach was adopted to explore whether the synergistic interaction on gene expression modulation between pemetrexed and gemcitabine is reproducible in humans and to determine the most meaningful interval to administer the two drugs. The expression of the main transporter and the key enzyme in the rate-limiting step of gemcitabine activation were measured in lymphocytes, at various intervals up to 48 hours, after pemetrexed administration, to explore whether the administration of this drug could induce an increase in their levels, suggesting a synergism between pemetrexed followed by gemcitabine, and to evaluate whether there was a reproducible timing of maximum dCK and hENT1 expressions to be used as the most appropriate interval between the administration of the two drugs.

The study confirmed the modulation of the dCK and hENT1 expressions induced by pemetrexed, showing a statistically significant increase of both variables at 1, 2, 24, and 48 hours from pemetrexed administration, irrespective of the cycle and of the 3- or 2-week schedule. Baseline values of both hENT1 and dCK were increased at 1 hour, in all patients.

Before study initiation, the specific gene expression was also quantified in six healthy subjects, showing baseline dCK and hENT1 levels, not being altered over the predefined time intervals (throughout 48 hours). These evidences supported the idea that the changes in gene expression observed in patients with NSCLC were specifically related to pemetrexed administration.

Moreover, univariate explorative regression analysis, which examined the potential relationship of dCK and hENT1 baseline values, and both peak values, with many different variables (e.g., demographic characteristics, dose of study drugs, tumor response, vital signs, and laboratory parameters) did not show significant correlation, demonstrating that changes in gene expression were not determined by patient clinical characteristics.

The activation peak, occurring at 1 to 2 hours after pemetrexed infusion, is aligned with the results of the previous randomized, phase II trial where pemetrexed on day 1, followed 90 minutes later by gemcitabine on days 1 and 8, was less toxic and met the protocol-defined efficacy criteria.[12] Furthermore, the 24-hour peak after pemetrexed administration supports an activation pattern similar to the modulation of dCK and hENT1 gene expression underlying the synergistic cytotoxicity of the pemetrexed→gemcitabine combination in NSCLC cells.[7]

Although the extrapolation of in vitro data to the in vivo setting should be considered with caution, these studies suggested that even small changes in gene expression/activity could make a difference in drug efficacy, and our pilot clinical trial should prompt further clinical studies to evaluate

whether these changes can, at least in part, explain the difference between the success and the failure of various chemotherapy schedules.

Therefore, these results suggested a time-related genetic synergism and do not support the suggestion to omit the delay between the infusion of pemetrexed and gemcitabine, which was just based on the absence of pharmacokinetic interaction between gemcitabine and pemetrexed when administered in rapid sequence.[21,22] Nevertheless, the present research is limited by its exploratory nature and the investigation of the changes in gemcitabine uptake, and intracellular activation would require further experiments and the study of how these changes might improve drug efficacy would need further clinical trials.

Preclinical and clinical studies show how dexamethasone might result in altered gene expression[23,24] and reduce sensitivity of lung cancer cells to gemcitabine.[25] In the current experience, the modulation of the dCK and hENT1 expressions, induced by pemetrexed, was highly reproducible among all 19 patients, although receiving dexamethasone premedication, as mandatory in clinical practice.

In conclusion, this is the first evidence of dCK and hENT1 induction by pemetrexed in humans, suggesting that the activity of the sequence pemetrexed→gemcitabine should be optimized by the administration of gemcitabine 1 to 2 or 24 to 48 hours after pemetrexed. These results support further studies to validate the role of dCK/hENT1 in vivo modulation for the optimization of gemcitabine-pemetrexed combination in patients with NSCLC.

Although limited by its exploratory nature, this study is an example of how a pharmacogenetic approach may offer the possibility of optimizing drug combinations, providing with appropriate information to further explore the most promising regimens in future randomized controlled trials.

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